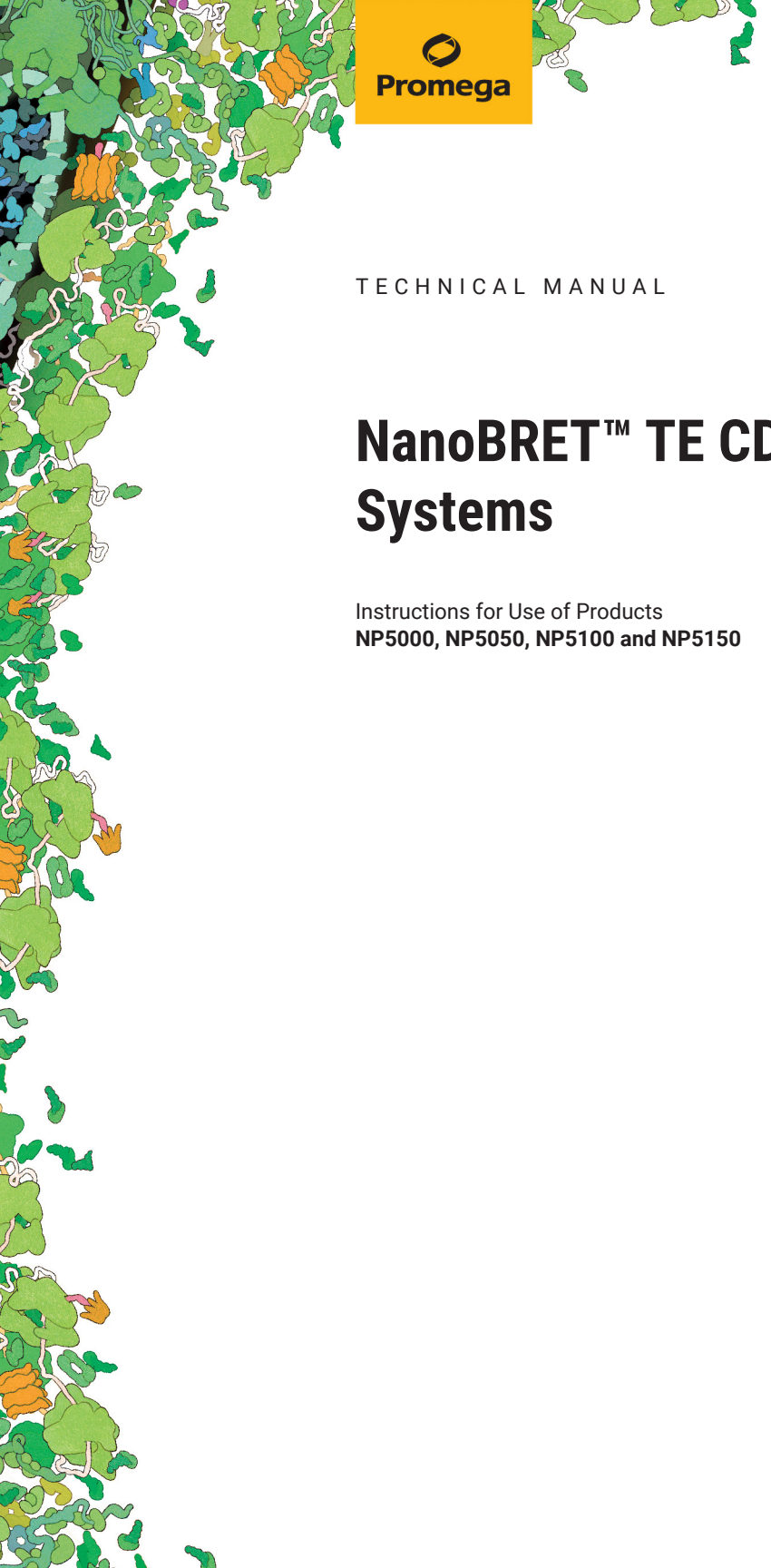

TECHNICAL MANUAL

NanoBRET™ TE CDK Selectivity Systems

Instructions for Use of Products
NP5000, NP5050, NP5100 and NP5150

9/23
TM684

NanoBRET™ TE CDK Selectivity Systems

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Various members of the Cyclin Dependent Kinase (CDK) family have been validated as oncogenic drivers in a variety of tumor types, and have therefore emerged as the most heavily pursued kinase subfamily in drug discovery. However, achieving isozyme selectivity with CDK inhibitors represents a major challenge based on the structural conservation within this critical kinase subfamily. Although there are biochemical assays for assessing inhibitor selectivity against a few well-studied CDK family members, live-cell approaches that systematically profile inhibitor selectivity across the majority of the CDK family have been lacking. Consequently, many CDK inhibitors fail to maintain their selectivity profile when evaluated in live cells. The NanoBRET™ Target Engagement (TE) CDK Selectivity Systems[a–j] directly address these challenges and enable researchers to profile inhibitor selectivity in live cells across a representative collection of CDKs in a simple workflow that can be performed in most high-throughput screening (HTS) laboratories.

NanoBRET™ TE CDK Selectivity Systems are based on NanoBRET™ TE technology, a bioluminescence resonance energy transfer (BRET) method that uses a small, extremely bright luciferase called NanoLuc (Figure 1). The NanoBRET™ TE technology quantitatively measures compound binding affinity and occupancy at select target proteins within live intact cells (1). It has been successfully applied to many target classes such as kinases (2–4). NanoBRET™ TE technology has also been applied to the entire CDK family, where a corresponding cyclin/regulatory protein is co-expressed along with the CDK-NanoLuc® fusion to create a biased readout for a specific CDK-partner protein pair (4). Target engagement can be quantified at 16 representative CDK-partner protein pairs using a single broad-spectrum NanoBRET™ Tracer, K-10, which enables a simplified live-cell CDK profiling method.

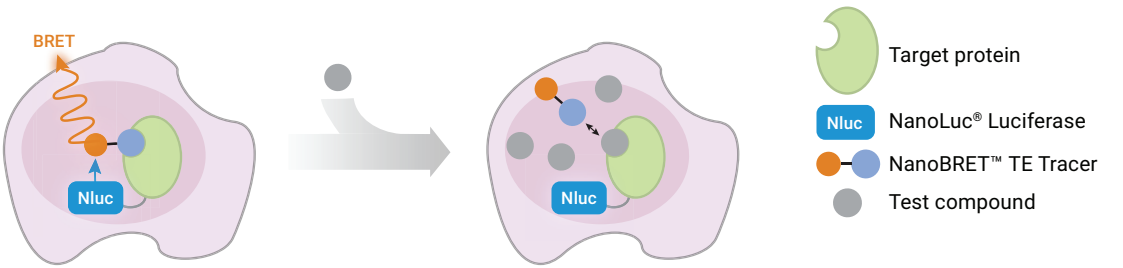

Figure 1. NanoBRET™ TE Assay Technology. The target of interest is expressed in mammalian cells as a target-NanoLuc® fusion construct. Compound engagement is measured in a competitive format using a cell-permeable fluorescent tracer. BRET is achieved by luminescent energy transfer from NanoLuc® luciferase to the cell-permeable fluorescent tracer that is bound to the target-NanoLuc® fusion protein. A test compound that is cell-permeable and competes with the tracer for target binding will result in a loss of NanoBRET™ signal. Additional details about the fundamental biophysical principles underlying the NanoBRET™ TE technology can be found in the *NanoBRET™ Target Engagement Intracellular Kinase Assay, Adherent Format Technical Manual* #TM598.

The NanoBRET™ TE CDK Selectivity Systems provide a novel microplate-based workflow that determines live-cell compound occupancy across a panel of CDKs in a single experiment, using common laboratory equipment. Two CDK selectivity systems are available that profile up to 16 CDKs in a single experiment in a customizable and modular format. The systems include one of two NanoBRET™ TE CDK Vector Panels[a] (A or B), each containing eight unique CDKs, as well as the NanoBRET™ TE Intracellular Kinase Assay, K-10[b–d] (Figure 2).

- The NanoBRET™ TE CDK Vector Panels A and B each contain eight unique CDK-NanoLuc® luciferase fusion vectors, with highly studied CDKs in Panel A (CDKs 1–7 and CDK9) and with understudied CDKs in Panel B (CDK10, CDKs 14–18, CDK20 and CDKL2). These are premixed with a corresponding cyclin/partner protein expression vector and only require dilution with TE buffer to prepare a convenient transfection-ready DNA mixture. On day 1 of the experiment, the user assembles the desired CDK vectors into a panel and transfects them into HEK293 cells. Each kinase/partner protein expressed by a vector within these panels is full-length.
- The NanoBRET™ TE Intracellular Kinase Assay, K-10 contains the necessary reagents to run the CDK NanoBRET™ TE assays in a single experiment on day 2. Tracer K-10 enables a single-reagent approach for CDK profiling and can be used at a single concentration to provide a quantitative assay for all 16 CDKs within the two panels. A test compound that competes with tracer for binding to the CDK will result in a loss of BRET signal. If desired, controls can be used to calibrate the BRET competition to calculate fractional occupancy for the test compound. The assay can be used to profile fractional occupancy of a test compound at a single dose or in a dose-response format to determine binding potency.

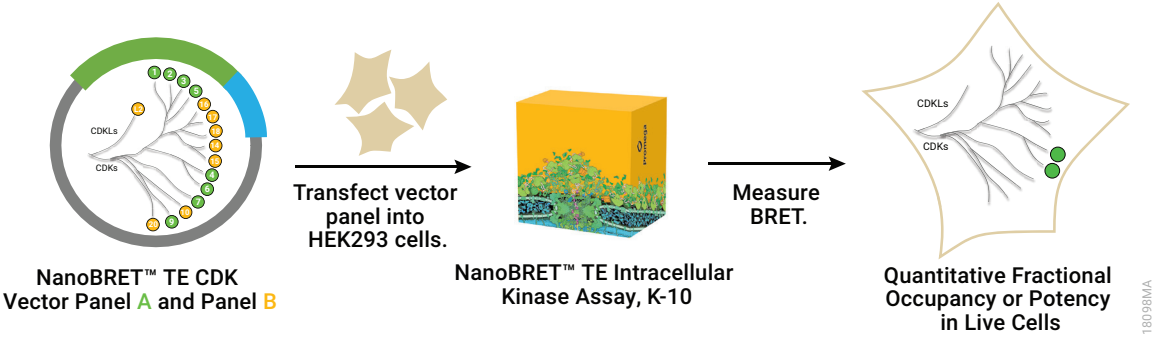

Figure 2. Overview of the NanoBRET™ Target Engagement CDK Selectivity Systems. Determining selectivity against a panel of up to 16 full-length CDKs in live cells using these assay systems. On day 1, the transfection-ready kinase vectors in the NanoBRET™ TE CDK Vector Panel A or NanoBRET™ TE CDK Vector Panel B are transfected into HEK293 cells in assay plates. On day 2, the NanoBRET™ TE Intracellular Kinase Assay, K-10 is performed, using NanoBRET™ Tracer K-10 to form a BRET complex with each kinase in the panel. A single tracer concentration of 1µM is used to provide quantitative data for each CDK. Competition for the BRET signal using a single dose of test compound determines fractional occupancy of the compound at each kinase within the panel. BRET signal competition using a dilution series of the test compound determines binding potency.

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1. Description (continued)

The NanoBRET™ method has been used in a case study to profile a collection of 46 clinically-advanced CDK inhibitors and tool molecules in live HEK293 cells (4). In this study, our results not only corroborated the behavior of advanced clinical compounds such as CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib, but also uncovered previously unappreciated drug-target interactions that offer potential drug repurposing opportunities for lesser-studied family members. These findings highlight the importance of determining drug selectivity comprehensively across the CDK family in live cells, an objective accomplished using the NanoBRET™ TE CDK Selectivity Systems.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ TE CDK Selectivity System A	**1 each**	**NP5050**

Each kit contains enough DNA for 120 analyses for each of the 8 CDK-NanoLuc® fusions. Includes:

- 1 each NanoBRET™ TE CDK Vector Panel A
- 1 each NanoBRET™ TE Intracellular Kinase Assay, K-10 (1,000 assays)

PRODUCT	SIZE	CAT.#
NanoBRET™ TE CDK Selectivity System B	**1 each**	**NP5150**

Each kit contains enough DNA for 120 analyses for each of the 8 CDK-NanoLuc® fusions. Includes:

- 1 each NanoBRET™ TE CDK Vector Panel B
- 1 each NanoBRET™ TE Intracellular Kinase Assay, K-10 (1,000 assays)

Available Separately

PRODUCT	SIZE	CAT.#
NanoBRET™ TE CDK Vector Panel A	**1 each**	**NP5000**
NanoBRET™ TE CDK Vector Panel B	**1 each**	**NP5100**

Storage Conditions: Store the NanoBRET™ TE CDK Vector Panels at –30°C to –10°C. Store the entire NanoBRET™ TE Intracellular Kinase Assay, K-10, at less than –65°C. Alternatively, store the NanoBRET™ Tracer K-10 at less than –65°C and all other components at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the vectors. We recommend aliquoting NanoBRET™ Tracer K-10 and avoiding more than five freeze-thaw cycles. Store the NanoBRET™ Tracer K-10, NanoBRET™ Nano-Glo® Substrate and Extracellular NanoLuc® Inhibitor protected from light.

3. Before You Begin

3.A. NanoBRET™ TE CDK Vector Panels A and B

There are eight CDK-NanoLuc® fusion vectors provided in each of the two selectivity systems (Panels A and B), including highly-studied CDKs in NanoBRET™ TE CDK Vector Panel A and understudied CDKs in NanoBRET™ TE CDK Vector Panel B. The 16 CDK-NanoLuc® fusion vectors provided across the two selectivity systems are in an easy-to-use, consumable formulation. The CDK-NanoLuc® fusion vectors are premixed with a corresponding cyclin or partner protein expression vector, avoiding the work of preparing individual mixtures of CDK-NanoLuc® fusion and partner protein vectors that are needed for optimal transfection and expression. These DNA mixtures are provided in individual tubes so that they can be tested in a modular and customizable fashion. The CDK-partner protein combinations within each of Panels A and B are described in Table 1.

Table 1. CDK Vector Panels.

CDK	CDK Fusion Vector	Partner Protein Expression Vector or Carrier DNA	Panel
CDK1	CDK1-NanoLuc® Fusion Vector	CCNB1	A
CDK2	CDK2-NanoLuc® Fusion Vector	CCNE1	A
CDK3	CDK3-NanoLuc® Fusion Vector	CCNE1	A
CDK4	NanoLuc®-CDK4 Fusion Vector	CCND3	A
CDK5	CDK5-NanoLuc® Fusion Vector	CDK5R1	A
CDK6	NanoLuc®-CDK6 Fusion Vector	CCND1	A
CDK7	NanoLuc®-CDK7 Fusion Vector	Transfection Carrier DNA[1]	A
CDK9	NanoLuc®-CDK9 Fusion Vector	CCNK	A
CDK10	CDK10-NanoLuc® Fusion Vector	CCNL2	B
CDK14	CDK14-NanoLuc® Fusion Vector	CCNY	B
CDK15	NanoLuc®-CDK15 Fusion Vector	CCNY	B
CDK16	CDK16-NanoLuc® Fusion Vector	CCNY	B
CDK17	CDK17-NanoLuc® Fusion Vector	CCNY	B
CDK18	CDK18-NanoLuc® Fusion Vector	CCNY	B
CDKL2	NanoLuc®-CDKL2 Fusion Vector	Transfection Carrier DNA[1]	B
CDK20	NanoLuc®-CDK20 Fusion Vector	CCNH	B

[1]No partner proteins are included in the CDK7 and CDKL2 DNA mixtures, but rather these include transfection carrier DNA to optimize transfection.

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3.B. NanoBRET™ TE Intracellular Kinase Assay, K-10

Performing this assay requires the following reagents (and the controls listed in Section 3.C):

- NanoBRET™ Tracer K-10
- NanoBRET™ Nano-Glo® Substrate
- Extracellular NanoLuc® Inhibitor
- Tracer Dilution Buffer

These reagents are provided in the NanoBRET™ TE Intracellular Kinase Assay, K-10 (Cat.# N2641), which is a component of the NanoBRET™ TE CDK Selectivity System A (Cat.# NP5050) and the NanoBRET™ TE CDK Selectivity System B (Cat.# NP5150).

3.C. NanoBRET™ TE Selectivity DNA Controls

If calculating test compound fractional occupancy is desired, we recommend including specific control vectors, which are found within the NanoBRET™ TE Selectivity DNA Controls[d,e] set. The set includes: 1) the NanoLuc® control vector; 2) the transfection control vector (NanoLuc®-HIPK2 Fusion Vector); and 3) Transfection Carrier DNA. The NanoBRET™ TE Selectivity DNA Controls are available separately (Cat.# NP1000). The preparation and use of these controls are described in Sections 4.D–4.F.

Note: The NanoLuc® control vector is the same vector as the pNL1.1.CMV(Nluc/CMV) Vector (Cat.# N1091), which is available separately.

3.D. Cell Background

The NanoBRET™ TE CDK Selectivity Systems were optimized in HEK293 cells. This assay panel has been validated specifically for use in either HEK293 cells (ATCC, Cat.# CRL-1573) or ready-to-use TransfectNow™ HEK293 Cells (Cat.# NC1001, NC1002). The NanoBRET™ TE CDK Selectivity Systems have not been validated for use in any other cell type.

3.E. Instrument Requirements and Setup

To perform NanoBRET™ TE Assays, a luminometer capable of measuring dual-wavelength windows is required. This is accomplished using filters. We recommend using a band pass (BP) filter for the donor signal and a long pass (LP) filter for the acceptor signal to maximize sensitivity.

- NanoBRET™ bioluminescent donor emission occurs at 460nm. To measure this donor signal, we recommend a band pass (BP) filter that covers close to 460nm with a band pass range of 8–80nm. For example, a 450nm/BP80 will capture in the 410–490nm range.
  Note: A BP filter is preferred for the donor signal measurement to selectively capture the signal peak and avoid measuring any acceptor peak bleedthrough. However, a short pass (SP) filter that covers the 460nm area also can be used. This may result in an artificially large value for the donor signal and measuring the bleedthrough into the acceptor peak, which could compress the ratio calculation and reduce the assay window.
- NanoBRET™ acceptor peak emission occurs at approximately 590–610nm. To measure the acceptor signal, we recommend a long pass filter starting at 600–610nm.

Instruments capable of dual-luminescence measurements are either equipped with a filter selection or the filters can be purchased and added separately. For instruments using mirrors, select the luminescence mirror. An integration time of 0.2–1 second is typically sufficient. Ensure that the gain on the instrument is optimized to capture the highest donor signal without reaching instrument saturation.

Consult with your instrument manufacturer to determine if the proper filters are installed or for the information needed to add filters to the luminometer. For example, a special holder or cube might be required for the filters to be mounted, and the shape and thickness may vary among instruments. We have experience with the following instruments and configurations:

- GloMax® Discover System (Cat.# GM3000) with preloaded filters for donor 450nm/8nm BP and acceptor 600nm LP. Select the preloaded BRET:NanoBRET™ 618 protocol from the 'Protocol' menu.
- BMG Labtech CLARIOstar® with preloaded filters for donor 450nm/80nm BP and acceptor 610nm LP.
- Thermo Varioskan® with filters obtained from Edmunds Optics, using donor 450nm CWL, 25mm diameter, 80nm FWHM, Interference Filter and acceptor 1 inch diameter, RG-610 Long Pass Filter.

Another instrument capable of measuring dual luminescence is the PerkinElmer EnVision® Multilabel Reader. To use the EnVision® for NanoBRET™ Assays, Perkin Elmer supplies the following optics:

- EnVision Optimized Label-NanoBRET (PE Cat.# 2100-8530). It includes: dichroic mirror, 585nm (Cat.# 2100-4380); filter, 460/80nm (Cat.# 2100-5950); and filter 647/75nm (Cat.# 2100-5970).

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4. NanoBRET™ TE CDK Selectivity Assay Protocols

Details for additional materials required for the assay that need to be obtained independently are listed in Materials to Be Supplied By the User. Sections 4.A and 4.B discuss treatment conditions and controls required for assay execution. Sections 4.C and 4.D provide details on preparing the working CDK panel DNAs and control DNAs, respectively. Sections 4.E–H provide details for the transfection and execution of the BRET assay, as well as guidance on data processing and quality control. Section 5.B lists the composition of required buffers and solutions.

Note: The volumes specified for the NanoBRET™ Target Engagement Protocols below are for 96-well plates. This protocol has not been optimized for use in 384-well plates.

Materials to Be Supplied By the User

- HEK293 cells (e.g., ready-to-use TransfectNow™ HEK293 Cells, Cat.# NC1001; or prepared-by-user, ATCC, Cat.# CRL-1573)
- Dulbecco's Modified Eagles Medium (DMEM; Thermo Fisher Scientific Cat.# 11995-065)
- fetal bovine serum (HyClone Cat.# SH30070.03 or Seradigm Cat.# 1500-050)
- Opti-MEM™ I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- white, tissue-culture treated 96-well plates (Corning® Cat.# 3917 is preferred; Greiner Cat.# 655083, alternate)
- polypropylene plasticware for tracer handling (**Note:** Polystyrene plasticware is not recommended for this assay.)
- 0.05% Trypsin/EDTA (Thermo Fisher Scientific Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311; **Note:** The transfection protocols used in this assay have been optimized using FuGENE® HD and may not be compatible with other transfection reagents.)
- DMSO (Sigma Cat.# D2650)
- NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000)
- TE Buffer, 1X, Molecular Biology Grade (Cat.# V6231)
- detection instrument capable of measuring NanoBRET™ wavelengths (i.e., GloMax® Discover System, Cat.# GM3000; see Section 3.E)
- **optional:** CC1 pan-Kinase Inhibitor (control ligand; Cat.# N2661)

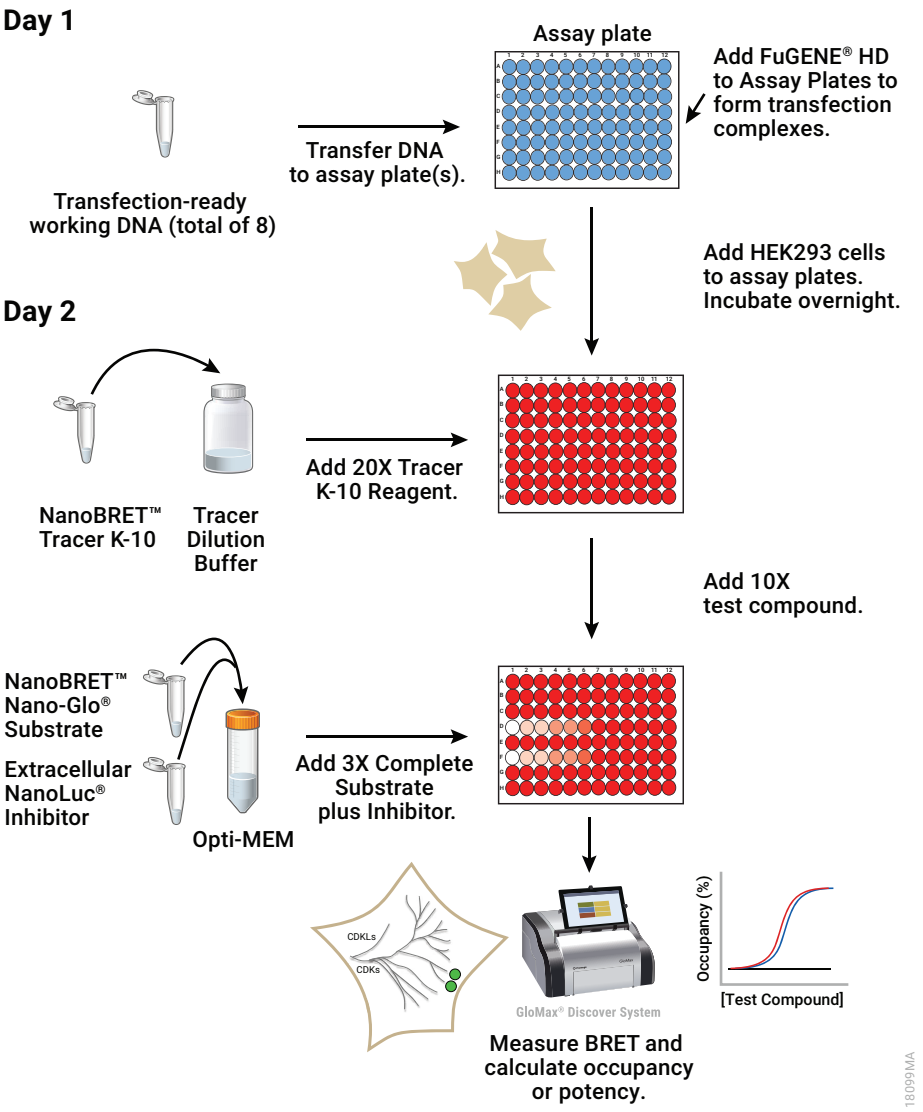

Figure 3. Schematic of the NanoBRET™ TE CDK Selectivity System protocol.

4.A. Required Conditions

The NanoBRET™ TE CDK Selectivity Assays can be used to assess both test compound fractional occupancy and affinity. If fractional occupancy for a test drug at a specific dose is the desired output, the following controls or conditions are required:

$BRET_{Min}$ Control: This control must be included in each experiment to define zero BRET or "100% Fractional Occupancy". The most efficient option for this control is to express the NanoLuc® control vector in cells and measure the BRET value. Adding the K-10 Tracer is not required. Specifically, the vector we recommend using as the NanoLuc® control is contained in the NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000) and is labeled pNL1.1.CMV(Nluc/CMV) Vector.

The $BRET_{Min}$ Control that expresses the NanoLuc® control vector can be placed in multiple wells on each assay plate, or it minimally needs to be placed in several wells on one of the assay plates. We recommend a minimum of two wells for the NanoLuc® control vector in each experiment. Preparation of the NanoLuc® control vector for transfection is detailed in Section 4.D.

Another option for the $BRET_{Min}$ Control is to measure the BRET value for all CDK assays in the presence of a saturating dose of test compound that can fully compete the BRET signal from the K-10 Tracer. We recommend a 20µM dose of CC1 pan-kinase Inhibitor (Cat.# N2661) for this purpose.

$BRET_{Max}$ Control: This control is needed for each kinase in the CDK panels. Specifically the $BRET_{Max}$ Control is used to define the maximum BRET or "0% Fractional Occupancy" for each CDK, and uses the K-10 Tracer + DMSO vehicle (or test compound solvent). Use of this control is detailed in Sections 4.G–H to perform data quality control and to calculate test compound fractional occupancy.

Sample: This condition is tracer + test compound for each CDK. This can be a single dose for calculation of fractional occupancy, or may be multiple test compound doses if using the assay to measure drug potency.

4.B. Recommended Conditions

The following controls or conditions are recommended:

Donor Background Control: This control is assay medium only and is used in combination with the detection reagents to measure the instrument background luminescence in the donor channel, to calculate donor signal-to-background (S/B) ratio for each CDK. Adding the K-10 Tracer is not required. We recommend including this control in technical duplicate in each experiment in isolated wells to minimize well-to-well bleedthrough from the NanoLuc® signal in nearby wells.

Transfection Control: This control is used to confirm that transfection was successful prior to executing the experiment on the full CDK panel, which can save time and expense in the event the transfection failed. Adding the K-10 Tracer is not required. The vector we recommend using as the Transfection Control is NanoLuc®-HIPK2 Fusion Vector, a component of NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000).

We recommend that a separate plate be prepared that includes the Transfection Control and Donor Background Control, and treating this plate identically to the CDK DNA panel. Transfect at least two wells for the Transfection Control and include at least two wells of the Donor Background Control in isolated wells to minimize well-to-well signal bleedthrough from the NanoLuc® signal in the transfection control wells that may compromise the signal-to-background calculation.

Note: Preparing the Transfection Control vector for use is detailed in Section 4.D. An example plate map for execution of the transfection control experiment is provided in Figure 4.

4.C. Preparation of Transfection-Ready Working DNA

The NanoBRET™ TE CDK Vector Panels A and B are formulated as consumable DNA mixtures in separate tubes, allowing these selectivity panels to be customized. DNA mixtures are 10X stocks containing the CDK-NanoLuc® fusion vector premixed with a corresponding cyclin or partner protein expression vector. Dilute the DNA provided to the concentration used for transfection. Once diluted, the transfection-ready working DNA can be stored and re-used multiple times for subsequent experiments. We recommend the following procedure to generate transfection-ready working DNA solutions.

1. Thaw NanoBRET™ TE CDK Vector Panel DNAs and centrifuge briefly to ensure all liquid is at the bottom of the tubes.
2. Add 1,080µl of sterile, nuclease-free TE buffer to each tube and mix gently. These samples are now the transfection-ready working DNA stocks.

   Notes:

   a. Change tips in between each TE buffer dispense to avoid cross contamination.

   b. If using the transfection-ready working DNA immediately to transfect cells, proceed to Section 4.E. If you won't be transfecting cells at this time, store at –20°C.

   c. For transfection-ready working DNA, we recommend limiting the number of freeze-thaws to five or fewer for best performance.

4.D. Preparation of Control Vectors for Transfection

All control vectors, such as the NanoLuc® control vector and the transfection control vector, are supplied in the NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000). Each vector is supplied in purified form at 1mg/ml. The NanoLuc® control and transfection control vectors must be mixed with Transfection Carrier DNA (Cat.# NP1000) then diluted to a working concentration before use.

Prepare the NanoLuc® Control Vector

1. Combine 1 part pNL1.1.CMV(Nluc/CMV) Vector (1mg/ml) with 9 parts Transfection Carrier DNA (1mg/ml) to prepare a 50X DNA mixture with a DNA concentration of 1mg/ml.
2. Dilute the 50X DNA mixture 1:50 in nuclease-free TE buffer to prepare the transfection-ready working solution with a DNA concentration of 20µg/ml.

   Note: Store the 50X DNA mixture in Step 1, and the transfection-ready working DNA solution in Step 2 at –20°C between uses. We recommend limiting the number of freeze-thaws to five or less for the transfection-ready working DNA solution for best performance.

Prepare the Transfection Control Vector

3. Combine 1 part NanoLuc®-HIPK2 Fusion Vector (1mg/ml) with 9 parts Transfection Carrier DNA (1mg/ml) to prepare a 50X DNA mixture with a total DNA concentration of 1mg/ml.
4. Dilute the 50X DNA mixture 1:50 in nuclease-free TE buffer to prepare the transfection-ready working solution with a total DNA concentration of 20µg/ml.

   Note: Store the 50X DNA mixture in Step 3, and the transfection-ready working DNA solution in Step 4 at –20°C in between uses. We recommend limiting the number of freeze-thaw cycles to five or less for the transfection-ready working DNA solution for best performance.

4.E. Transfection Workflow (Day 1)

This workflow allows preparation of transfection complexes directly in the wells of the 96-well white assay plates. From an efficiency standpoint, we recommend first setting up the transfection complexes in Steps 2–7 below. While the transfection complexes are incubating, proceed to Step 8 to harvest the HEK293 cells. For large-scale experiments, we recommended performing these steps with two scientists: one to prepare the transfection complexes and the other to prepare the cell suspension. TransfectNow™ HEK293 Cells can simplify the workflow and avoid the need to routinely culture, expand and harvest HEK293 cells. If using TransfectNow™ HEK293 Cells, thaw the cells and resuspend in assay medium as described in Step 9 below. Consult the *TransfectNow™ HEK293 Cells Technical Manual* #TM690 for complete details on the use of these cells.

1. Passage HEK293 cells on the day prior to transfection such that the cells are 75–95% confluent on the day of transfection.

   Note: If using TransfectNow™ HEK293 Cells, skip Step 1 and start with Step 2.

2. Estimate the required volume of transfection complex by determining the number of data points needed for each CDK including the desired number of technical replicates and the required and recommended controls from Section 4.D. For example, testing an 11-point dilution series of a test compound in technical singlicate would require 11 wells of analysis for each CDK.

   Notes:

   a. If calculating fractional occupancy (Section 4.A), this experiment would also require a twelfth well for each CDK as the $BRET_{Max}$ Control (test compound solvent), as well as a minimum of 2 wells for the $BRET_{Min}$ Control (NanoLuc® control vector).

   b. If performing a Transfection Control experiment (Section 4.B), we recommend a minimum of technical duplicates for both the Transfection Control and the Donor Background Control. These should be set up in a separate assay plate, such that these wells are isolated in the assay plate to prevent signal bleedthrough that can compromise the signal-to-background calculation. An example plate layout for the Transfection Control is provided in Figure 4.

3. Transfer 10µl of each transfection-ready working DNA solution to the desired assay well.

   Notes:

   a. If the transfection-ready working stocks are frozen, we recommend thawing the DNA quickly at 37°C. Mix gently on an orbital shaker and then centrifuge the tube briefly to ensure the solution is at the bottom of the vessel. Each assay well requires 10µl of the transfection-ready working DNA solution.

   b. Add the DNA solutions to the bottom corner of the well (rather than the center) for most efficient complex formation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

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Figure 4. Example plate layout for the transfection control experiment.

4. Prepare a 20µl/ml FuGENE® HD solution by diluting FuGENE® HD to a final concentration of 20µl/ml in room temperature Opti-MEM™ medium in a sterile conical tube. Pipet directly into the liquid, being careful not to touch the sides of the plastic tube.

   Note: For each analysis well, prepare 30µl of the diluted FuGENE® HD solution. For example, testing an 11-point test compound dilution series against all 16 CDKs from panels A and B requires a total of 176 wells and thus 5.28ml of 20µl/ml FuGENE® HD solution in Opti-MEM™ medium. If calculating fractional occupancy and including all required or recommended controls, this experiment would require 196 wells: 192 wells for the 16 CDKs (which includes an eleven-point sample plus $BRET_{Max}$ Control per CDK); 2 wells for the $BRET_{Min}$ Control (NanoLuc® control vector), and; 2 wells for the transfection control vector, for a total of 5.88ml of the 20µl/ml FuGENE® HD solution in Opti-MEM™ medium. Remember to prepare extra to account for dead volume.

5. Add 30µl of 20µl/ml Fugene® HD solution to each assay well containing the 10µl of transfection-ready DNA.

   Note: Add the FuGENE® HD solution on the side of the well where the DNA solutions were added previously for most efficient complex formation. Avoid directly touching pipette tips to the DNA solution to prevent cross contamination.

6. Mix assay plate(s) on an orbital shaker for 15 seconds at 500–600rpm.

7. Incubate for 30 minutes at room temperature to allow complexes to form.

8. While the complexes are forming in Step 7, prepare an HEK293 cell suspension. If you've propagated HEK293 cells in your laboratory, follow these steps to prepare them for assay. Alternatively, if using TransfectNow™ HEK293 Cells, proceed to Step 9 for instructions.

   a. Trypsinize the cells, then inactivate the trypsin using cell culture medium (see Section 5.B).

   b. Centrifuge the cells at 200 × *g* for 3–5 minutes to pellet, then aspirate the supernatant.

   c. Resuspend the cells in assay medium (Section 5.B) at a density of 2.5×10^5 cells/ml.

   d. Proceed to Step 10.

9. **Optional:** While the complexes are forming in Step 7, follow these directions if using TransfectNow™ HEK293 Cells:
   a. Remove the required number of vials of TransfectNow™ HEK293 cells from storage at –140°C and transfer to the bench on dry ice.
   b. Add the appropriate volume of prewarmed (37°C) assay medium (Section 5.B) to a 50ml conical tube, depending on the size vial you are thawing. For 0.5ml vials, add 17ml of assay medium. For 1ml vials, add 34ml of assay medium.
   c. Warm the TransfectNow™ HEK293 Cells in a 37°C water bath until just thawed (1–2 minutes). While thawing, gently agitate and visually inspect. Do not submerge the vial completely. Do not invert the vial. After cells are thawed, dry vial and spray with 70% alcohol.
   d. Gently mix the cell suspension by pipetting, then transfer the cells to the 50ml conical tube containing assay media. Mix well by gently pipetting or inverting 1–2 times.
10. Add 60µl of the cell suspension per well to plates containing the transfection complexes from Step 7.
11. Incubate overnight at 37°C in a 5% CO_2 incubator.

    Note: Allow a minimum of 16 hours for transfection and expression of the transgenes to occur; 20–24 hours is ideal.

4.F. Transfection Control Experiment (Day 2)

1. Remove the Transfection Control plate from the incubator and allow to equilibrate to room temperature for 15 minutes.
2. Prepare 3X Complete Substrate plus Inhibitor Solution. This solution consists of a 1:166 dilution of NanoBRET™ Nano-Glo® Substrate plus a 1:500 dilution of Extracellular NanoLuc® Inhibitor in Opti-MEM™ medium without serum or phenol red. Mix gently by inversion 5–10 times in a conical tube. For example, for the transfection control experiment depicted in Figure 4, dilute 0.5µl of the Extracellular NanoLuc® Inhibitor and 1.5µl of NanoBRET™ Nano-Glo® Substrate to 250µl with Opti-MEM™ medium.

   Note: The 3X Complete Substrate plus Inhibitor Solution should be used within 1.5 hours of preparation.
3. To wells containing the transfection control or the Donor Background Control, add 50µl per well of the 3X Complete Substrate plus Inhibitor Solution. Incubate 2–3 minutes at room temperature.

   Note: If the Donor Background Control was not set up in advance as in Figure 4, add 100µl per well of assay medium and 50µl per well of Complete Substrate plus Inhibitor solution to wells on the opposite side of the plate from wells containing the Transfection Control.
4. Measure donor emission (e.g., 450nm) for both the Transfection Control and the Donor Background Control wells using a BRET-compatible luminometer.
5. Calculate the signal-to-background (S/B) ratio in the donor channel using the following equation:

   S/B ratio = X/Y

   where X = mean donor signal for the transfection control wells and Y = mean donor signal for the Background Control wells.

   Note: The S/B ratio should be >1,000 in order to proceed with the assay plates transfected on day 1. Ideally the S/B ratio is in the range of 10,000–100,000.

4.G. NanoBRET™ Target Engagement Assay Protocol (Day 2)

Prepare NanoBRET Tracer K-10 Reagents and Add to Cells

1. Based upon the treatment conditions and number of CDKs in the analysis, calculate the number of data points needed.

   Note: For example, if testing an 11-point dilution series of a test compound across all 16 CDKs and including controls for calculation of fractional occupancy, this experiment would require 194 wells (12 wells for each CDK plus 2 wells for the NanoLuc® control vector).

2. Prepare a 100X (100µM) solution of NanoBRET™ Tracer K-10 in DMSO. This is a fourfold dilution of the provided 400µM NanoBRET™ Tracer K-10 stock solution.

3. Dilute 1 part of the 100X solution with 4 parts of tracer dilution buffer to prepare the Complete 20X NanoBRET™ Tracer K-10 Reagent.

   Notes:

   a. Each well requires 5µl of the Complete 20X Tracer Reagent. For the example above with 194 analyses, this experiment would require 970µl of the Complete 20X Tracer Reagent. Remember to prepare extra reagent to account for dead volume.

   b. To prepare the Complete 20X NanoBRET™ Tracer K-10 Reagent, we recommend first adding the concentrated tracer stock and DMSO to a conical tube, then adding the Tracer Dilution Buffer. Mix and transfer the Complete 20X NanoBRET™ Tracer K-10 Reagent into polypropylene (not polystyrene) troughs for dispensing.

4. Add 5µl of Complete 20X NanoBRET™ Tracer Reagent per well to the transfected cells (directly into the liquid) according to the plate layout.

   Optional: If calculating fractional occupancy, add 5µl of Complete 20X NanoBRET™ Tracer Reagent to the $BRET_{Max}$ Control and any $BRET_{Min}$ Control wells that are executed by competing the BRET signal with a saturating dose of the CC1 pan kinase inhibitor. If using the NanoLuc® control vector for the $BRET_{Min}$ Control wells, adding the K-10 tracer is not required.

5. Mix on an orbital shaker for 15 seconds at 900rpm.

   Note: Mixing speed can vary between orbital shakers and should be optimized for each unit by visual inspection to ensure complete dispersal of the tracer.

Prepare 10X Test Compound or 10X $BRET_{Max}$ Control and Add to Cells

6. Dilute the test compound to 1,000X in DMSO (or the test compound solvent). If testing a compound dose response, prepare a 1,000X dilution series of the compound in DMSO (or test compound solvent).

7. Dilute the 1,000X test compound or test compound dilution series to 10X in Opti-MEM™ medium.
   Optional: If calculating fractional occupancy, include the $BRET_{Max}$ Control condition (DMSO or test compound solvent only). If using the NanoLuc® control vector for the $BRET_{Min}$ Control, also prepare a 10X vehicle solution by diluting test compound solvent 100-fold into Opti-MEM™ medium. If using a saturating dose of the CC1 pan-kinase inhibitor for the $BRET_{Min}$ Control, also prepare 200µM solution of CC1 in Opti-MEM™ medium.

4.G. NanoBRET™ Target Engagement Assay Protocol (Day 2; continued)

8. Add 10µl per well of the 10X test compound or dilution series.
   Optional: If calculating fractional occupancy, include the $BRET_{Max}$ Control condition. If using the NanoLuc® control vector for the $BRET_{Min}$ Control, add 10µl of the 10X vehicle solution to the $BRET_{Min}$ Control wells. If using a saturating dose of the CC1 pan-kinase inhibitor for the $BRET_{Min}$ Control, add 10µl of 200µM CC1 to the $BRET_{Min}$ Control wells.
9. Mix plate on an orbital shaker for 15 seconds at 900rpm.
10. Incubate the plate at 37°C in a 5% CO_2 incubator for 2 hours.
11. After the 2-hour incubation, allow plate to cool to room temperature for approximately 15 minutes.
12. Immediately prior to BRET measurements, prepare 3X Complete Substrate Plus Inhibitor solution. This solution consists of a 1:166 dilution of NanoBRET™ Nano-Glo® Substrate plus a 1:500 dilution of Extracellular NanoLuc® Inhibitor in Opti-MEM™ medium without serum or phenol red, combined in a conical tube. Mix gently by inversion 5–10 times.

    Notes:

    a. For the example above, there are a total of 194 wells of analysis including CDKs and the NanoLuc® control vector ($BRET_{Min}$ Control), requiring a minimum of 9.7ml of 3X Complete Substrate Plus Inhibitor solution. For convenience and to account for overage, prepare at least 10ml of 3X Complete Substrate Plus Inhibitor solution by diluting 20µl of Extracellular NanoLuc® Inhibitor and 60µl of NanoBRET™ Nano-Glo® Substrate to 10ml with Opti-MEM™ medium.

    b. The 3X Complete Substrate Plus Inhibitor solution should be used within 1.5 hours of preparation.
13. Add 50µl per well of 3X Complete Substrate Plus Inhibitor solution and incubate 2–3 minutes at room temperature.
14. Measure donor emission wavelength (e.g., 450nm) and acceptor emission wavelength (e.g., 610nm) using the Glomax® Discover System or another NanoBRET™ Assay-compatible luminometer (see Section 3.E. for details).

4.H. Data Quality Analysis and Fractional Occupancy

This section describes the workflow to perform data quality control analysis, calculate BRET ratios and fractional occupancy values, if desired. For an example of this data calculated for an individual kinase, see Section 5.E.

Donor Signal and Donor Signal-to-Background (Donor S/B)

Inspect the donor emission signal (e.g., 450nm) for each individual CDK and determine if expression was adequate in each well. Then, inspect the donor emission signal (e.g., 450nm) across the entire set of CDKs and determine if cumulative expression was adequate.

Note: Steps 1–3 require Donor Background Control samples. If you omitted this control, proceed to Step 4.

1. Calculate the mean donor signal for each CDK, or the average donor signal across all wells containing an individual CDK. Calculate mean donor signal for Donor Background Control wells.

2. Calculate the donor S/B for each individual CDK by dividing the mean donor signal for each CDK by the mean donor signal for the Donor Background Control wells.

   Note: Ideal donor S/B for most CDKs will be 10,000–100,000. Consider excluding CDKs with donor S/B less than 1,000.

3. Calculate the mean donor S/B across the entire set of CDKs by dividing the mean donor signal across all CDKs by the mean donor signal for the Donor Background Control wells.

   Note: Ideal donor S/B across the entire set of CDKs will be in the range of 10,000–100,000. If the donor S/B across the entire set of CDKs is <1,000 consider repeating the experiment.

BRET Signals

4. Calculate the raw BRET values for all wells by dividing the acceptor emission signal (e.g., 610nm) by the donor emission signal (e.g., 450nm)

Optional: Assay Window

5. Calculate the mean BRET values for the $BRET_{Max}$ Controls for each individual CDK (tracer + vehicle) and the $BRET_{Min}$ Controls (e.g., NanoLuc® control vector).

6. Calculate the assay window for each individual CDK assay by dividing the mean BRET value for the $BRET_{Max}$ Control for each CDK by the mean BRET value for the $BRET_{Min}$ Control.

   Note: When we execute this protocol using the GloMax® Discover Instrument, assay windows for the 16 CDKs are routinely above 2. See Section 5.A for example assay window data for each CDK. Occasionally, the assay window for some individual kinases may be in the range of 1.7–2 for normal executions. In our experience, an experiment in which more than 1 CDK has an assay window <2 is atypical and suggests that data quality for the entire experiment might be suspect. These ranges may be used as a starting point for quality control of individual experiments, but may need revision depending on the normal values obtained using your equipment and experimental setup, which may differ depending upon the HEK293 cells or plate reader used.

Optional: Fractional Occupancy

7. Calculate fractional occupancy for the test drug for each CDK using the following formula:

$$\text{Occupancy (\%)} = \left[1 - \frac{(\text{Sample} - BRET_{Min})}{(BRET_{Max} - BRET_{Min})} \right] \times 100$$

   Where:

   Sample = Mean BRET value across all sample (tracer + compound) wells for an individual CDK at a specific compound dose.

   $BRET_{Max}$ = Mean BRET value across all $BRET_{Max}$ (tracer + vehicle) control wells for an individual CDK.

   $BRET_{Min}$ = Mean BRET value of NanoLuc® control wells (calculated either on a plate-by-plate basis or across the entire experiment).

   Note: Alternatively, the $BRET_{Min}$ value may determined by treating samples with a saturating dose of a known control ligand and measuring the BRET value. We recommend the CC1 pan-Kinase Inhibitor at a dose of 20µM for this purpose, which fully competes with the BRET signal for all CDKs in Panels A and B.

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5. Appendix

5.A. Example Assay Window and Selectivity Data for Control Compounds

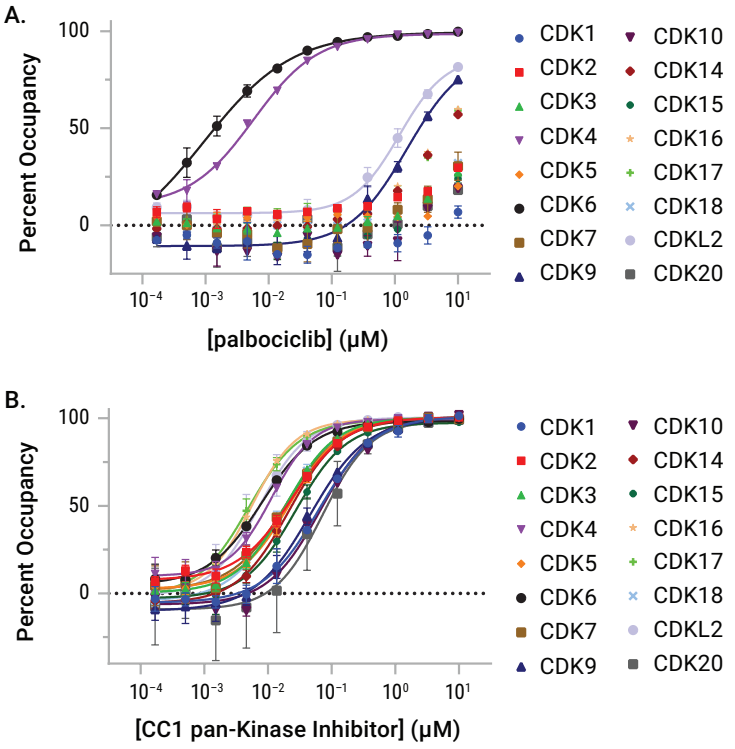
The NanoBRET™ TE CDK Selectivity Systems provide unique information regarding the intracellular selectivity of test compounds across a representative set of CDKs. However, due to the distance and geometry components of BRET, each individual CDK assay within the panel may exhibit a different assay window. Within this panel of up to 16 assays, the assay windows range from three- to thirteenfold, with a median assay window of approximately sixfold. We have observed consistent assay windows for each individual kinase assay across many experimental executions, with typical assay window coefficient of variation (CV) of approximately 9%. For some individual CDKs, the assay window may vary to a greater extent between experiments, but this variation generally does not impact the measurement of fractional occupancy for test compounds. Example assay windows are provided in Table 2.

Due to myriad factors present inside cells (e.g., intracellular ATP, protein complexes, variable kinase activation states, etc.) the selectivity behavior of test compounds observed in live cells can be very different compared to the selectivity behavior in cell-free kinase assays. To ensure that the NanoBRET™ TE Intracellular Kinase Selectivity Assay is performing properly, we recommend including control compounds. We have extensive experience testing two control compounds with unique selectivity behavior. These include the CC1 pan-Kinase Inhibitor, a promiscuous kinase ligand that binds to every CDK within the selectivity panel, and palbociclib, which demonstrates greater intracellular selectivity for CDK4 and CDK6. Occupancy data at specific doses for each of these control compounds is provided in Table 2, and example dose responses for these compounds are provided in Figure 5. To build confidence in experimental fidelity, we recommend initially testing both of these control compounds and comparing to the historical data in Table 2 for general agreement. After initial use, one or both of these controls can be used occasionally to calibrate new users or be included in each run as a routine internal control for expected assay behavior. Lastly, we have profiled the potency of a collection of 46 clinically-advanced CDK inhibitors and tool molecules in live HEK293 cells using a similar approach (4), and that data set can be used as resource for comparison while establishing this CDK selectivity assay internally.

Table 2. Individual CDK Assay Performance and Control Inhibitor Data. Data in this table were created using NanoBRET™ Tracer K-10 at 1,000nM. Inhibitors used to compete with kinases include CC1 pan-Kinase Inhibitor (300nM) and palbociclib (500nM). The CDK-partner protein combinations shown in Table 2 are described in detail in Table 1.

		Assay Window			CC1 pan-Kinase Inhibitor Occupancy (%)		Palbociclib Occupancy (%)	
Kinase	Partner Protein	Mean	Standard Deviation	CV (%)	Mean	Standard Deviation	Mean	Standard Deviation
CDK1	CCNB1	3.1	0.2	6.4	71	7.1	1	0.4
CDK2	CCNE1	12.2	0.8	6.3	80	4.0	1	3.1
CDK3	CCNE1	6.8	0.2	3.5	85	3.5	−1	1.4
CDK4	CCND3	7.3	1.7	23.5	93	2.2	95	2.1
CDK5	CDK5R1	13.4	1.0	7.1	85	3.6	3	2.6
CDK6	CCND1	4.3	0.4	8.8	89	2.2	96	1.6
CDK7	None	3.5	0.2	6.4	83	4.1	−2	3.0
CDK9	CCNK	4.8	0.4	7.5	77	0.3	23	12.4
CDK10	CCNL2	3.3	0.3	9.4	62	0.9	−12	33
CDK14	CCNY	4.3	0.2	4.7	82	1.3	7	1.1
CDK15	CCNY	3.1	0.2	6.3	78	2.1	2	0.9
CDK16	CCNY	7.0	0.3	4.7	96	0.6	14	9.2
CDK17	CCNY	9.4	0.4	4.7	95	0.8	12	8.2
CDK18	CCNY	10.9	0.2	1.8	88	2.1	1	4.2
CDKL2	None	7.2	0.9	13.0	95	1.3	22	10.7
CDK20	CCNH	5.1	1.0	20.4	54	4.9	−8	14.9

5.A. Example Assay Window and Selectivity Data for Control Compounds (continued)



C.

CDK	Panel	Palbociclib IC_{50} (nM)	CC1 pan-Kinase Inhibitor IC_{50} (nM)
CDK1	A	Weak Binding	63
CDK2	A	Weak Binding	24
CDK3	A	Weak Binding	19
CDK4	A	5.7	12
CDK5	A	Weak Binding	22
CDK6	A	0.95	8.0
CDK7	A	Weak Binding	19
CDK9	A	1600	46
CDK10	B	Weak Binding	66
CDK14	B	Weak Binding	21
CDK15	B	Weak Binding	27
CDK16	B	Weak Binding	5.6
CDK17	B	Weak Binding	4.9
CDK18	B	Weak Binding	17
CDKL2	B	1200	7.2
CDK20	B	Weak Binding	72

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Figure 5. Representative data for palbociclib and CC1 pan-Kinase Inhibitor in HEK293 cells transiently expressing both CDK selectivity panels. HEK293 cells expressing CDKs in Panels A and B were combined with 1µM NanoBRET™ Tracer K-10 and a dilution series of either palbociclib (**Panel A**) or CC1 pan-Kinase Inhibitor (**Panel B**). After a 2-hour incubation, Complete NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor was added and BRET measurements were made on a luminometer equipped with 450/80BP and 610/LP filters. Percent occupancy was calculated as described in this protocol, using the BRET signal in the presence of a saturating dose (20µM) of the CC1 pan-Kinase Inhibitor to calculate the $BRET_{Min}$ value. **Panel C.** Representative affinity (IC_{50}) values for the selective inhibitor palbociclib and the promiscuous CC1 pan-Kinase Inhibitor across the CDK panels.

5.B. Composition of Buffers and Solutions

cell culture medium

90% DMEM
10% fetal bovine serum

assay medium

99% Opti-MEM™ I Reduced Serum Medium, no phenol red
1% fetal bovine serum

5.C. Frequently Asked Questions

1. Can the NanoBRET™ TE CDK Selectivity Assays be performed in another cell type?

   Answer: Though technically the NanoBRET™ TE CDK Selectivity Assays could be performed in a cell type other than HEK293 cells, it would require revalidation of all individual kinase assays in the alternative cell line to ensure that the tracer concentrations used for each kinase remain quantitative. Moreover, there is no guarantee that each individual kinase will perform adequately in the alternative cell background. For these reasons, running the assays in another cell line is not practical.

2. Can I make transfection complexes for multiple assay wells outside of the assay plate (in bulk) and then dispense into the assay plate?

   Answer: Yes, see an example application using bulk transfection complexes in our STAR Methods Article (6).

3. Can I store working DNAs in strip tubes or 96-well plates instead of in individual tubes?

   Answer: Using transfection-ready working DNAs in a preformatted setup, such as in a strip tube or 96-well plate, may be more convenient. If the DNAs are being stored in 96-well plates, you should seal the plates tightly using aluminum sealing film (i.e., Axygen Aluminum Sealing Film, Corning Cat.# PCR-AS-600) or using a heat seal appropriate for the particular storage plate. We also recommend placing a clear 96-well assay plate lid on top of the sealed plate prior to storage at –20°C, as the lid will help keep the sealing film tightly attached and reduce evaporation. We have observed evaporation when appropriate plate sealing is not used.

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5.D. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Weak expression levels (mean 450nm donor signal-to-background <1,000)	Cell status at the time of transfection. Ensure that the cells were passaged on the day prior to transfection, and that the cell confluency was appropriate (75–95%). Alternatively, use TransfectNow™ HEK293 Cells.
	Inaccurate DNA working solution preparation. Ensure the DNA has been diluted to the working concentration and stored properly to reduce evaporation (Sections 4.C–D, 5.C).
	Inaccurate transfection complex preparation. Rely on the transfection control samples to ensure that each experiment results in appropriate transfection levels prior to executing the full CDK profiling experiment. This can save reagent when aberrant transfections occur.
Noisy assay window (BRET S/B), generating coefficient of variation (CV) >20%	Weak expression levels. Ensure that the donor (450nm channel) RLUs for each CDK are >1,000 above the Donor Background Control (Section 4.B).
	Inconsistent dispensing of tracer. Ensure that liquid handlers are accurately delivering the tracer to each well.
Negative fractional occupancy (percent) of test compound	Inaccurate dispensing of tracer for $BRET_{Max}$ Controls (100% BRET, or 0% fractional occupancy controls). Ensure liquid handler is accurately dispensing the NanoBRET™ tracer.
	Autofluorescent or light-scattering properties of the test compound. Optical effects may increase the BRET value. This is often determined by using an irrelevant BRET control assay. If the compound has the same effect on an irrelevant BRET assay, this is likely a spurious optical effect. Potential control assays are listed in Related Products, Section 5.G, and include BRET assays for HDAC, BET BRD, E3 ligases CRBN and VHL, or any other BRET assay for a target that should not bind the test compound.

Symptoms	Causes and Comments
Negative fractional occupancy (percent) of test compound	Although rare, global or nonspecific impacts on kinase activation state may be observed. Nonspecific kinase inhibitors may indirectly impact the target of interest, altering the activation state of the kinase. In some cases, altering the activation state may increase the apparent affinity of the NanoBRET™ tracer leading to a nonspecific increase in BRET. It may be possible to run specific NanoBRET™ CDK assays in digitonin-treated cells to determine if this increase in BRET is due to such nonspecific pathway influences as described in earlier studies (3,5).
Unexpectedly low fractional occupancy (percent) of test compound	Inaccurate dispensing of test compound. Ensure liquid handling is accurately dispensing the compound.
	Poor compound solubility. Ensure that the compound is soluble as a 10X solution.
	Discordance between a cell-free and live cell target engagement assay. If comparing NanoBRET™ to a cell-free assessment of target occupancy, consider the impact of permeability or [ATP], which may interfere with target engagement. The composite effect of these variables may shift the occupancy results in a live cell versus an acellular system. Follow up experiments in digitonin-treated cells may be warranted to address the impact of [ATP] or permeability as described in earlier studies (3,5).
Unexpectedly high fractional occupancy (percent)	Inaccurate dispensing of test compound. Ensure liquid handling of test compound is accurately dispensing the compound.
	Discordance between a cell-free and live-cell target engagement assay. If comparing NanoBRET™ to a cell-free assessment of target occupancy, consider the impact of target activation state. If the compound preferentially engages an active or inactive kinase state, this may impact intracellular engagement to an unpredictable extent.

5.E. Example Data Processing and Fractional Occupancy Calculation

Figure 6 shows example raw data for the CDK6 assay tested with a single dose of test compound (palbociclib), as well as the processing of that data to calculate fractional occupancy.

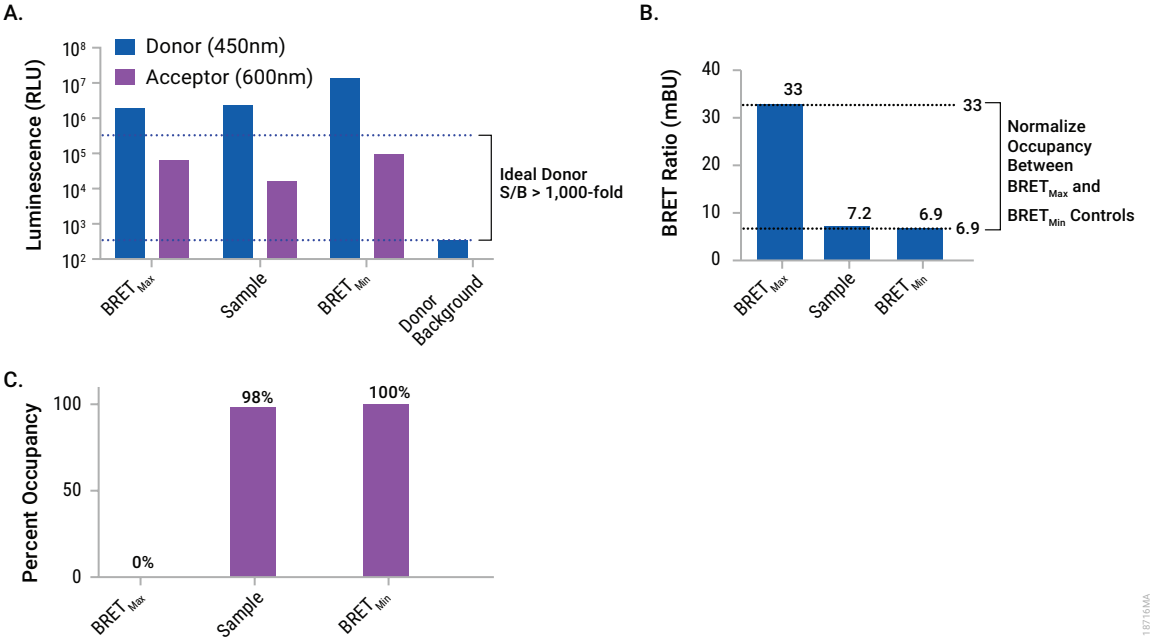

Figure 6. Example raw data and processing for the CDK6 assay. HEK293 cells expressing NanoLuc®-CDK6 and Cyclin D1 in a 96-well assay plate were combined with 1µM NanoBRET™ Tracer K-10 and treated with a sample (500nM palbociclib) or DMSO vehicle ($BRET_{Max}$ Control). The NanoLuc® control vector was included in the experiment as the $BRET_{Min}$ Control and a Donor Background Control was included to assess quality of the expression. After a 2-hour incubation, Complete NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor was added and BRET measurements were made on a luminometer equipped with 450/80BP and 610/LP filters. **Panel A.** Example raw donor (450nm) and acceptor (600nm) are plotted for both the $BRET_{Max}$ and $BRET_{Min}$ Controls, as well as the palbociclib-treated sample. The sample and controls demonstrated donor signals that were > 1,000-fold above the Donor Background Control. **Panel B.** BRET ratios were calculated as mBRET values for the $BRET_{Min}$ and $BRET_{Max}$ Controls as well as the palbociclib-treated sample. **Panel C.** Percent occupancy was calculated as described in this protocol, using the BRET values for $BRET_{Max}$, $BRET_{Min}$ and sample from **Panel B.**

5.F. Extinction Coefficient of NanoBRET™ Tracers

NanoBRET™ Tracer K-10 uses the NanoBRET™ 590 fluorophore. The concentration of NanoBRET™ Tracer K-10 was determined using an extinction coefficient of 83,000 M^{-1} cm^{-1} at 590nm. See Table 10.1 in reference (7) for details.

5.G. References

1. Machleidt, T. *et al.* (2015) NanoBRET-A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Bio.* **10**, 1797–804.
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3. Vasta, J.D. *et al.* (2018) Quantitative, wide-spectrum kinase profiling in live cells for assessing the effect of cellular ATP on target engagement. *Cell Chem. Biol.* **25**, 206–14.
4. Wells, C.I. *et al.* (2020) Quantifying CDK inhibitor selectivity in live cells. *Nat. Commun.* **11**, 2743.
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7. In: *Anthropological Genetics: Theory, Methods and Applications*, Michael H. Crawford, *ed.* (2006) University of Cambridge Press.

5.H. Related Products

NanoBRET™ TE K192 Kinase Selectivity System and Reagents

Product	Size	Cat.#
NanoBRET™ TE K192 Kinase Selectivity System	1 each	NP4050
NanoBRET™ TE K192 Kinase Selectivity System with Controls	1 each	NP4060
NanoBRET™ TE K192 Kinase Vector Panel	1 each	NP4100
NanoBRET™ TE K192 Kinase Vector Panel, Small	1 each	NP4101
NanoBRET™ TE Selectivity DNA Controls	1 each	NP1000
TransfectNow™ HEK293 Cells, 1 × 0.5ml	1 each	NC1001
TransfectNow™ HEK293 Cells, 2 × 1ml	1 each	NC1002

NanoBRET™ TE Intracellular Kinase Assays

Product	Size	Cat.#
NanoBRET™ TE Intracellular Kinase Assay K-3	100 assays	N2600
NanoBRET™ TE Intracellular Kinase Assay K-4	100 assays	N2520
NanoBRET™ TE Intracellular Kinase Assay K-5	100 assays	N2500
NanoBRET™ TE Intracellular Kinase Assay K-8	100 assays	N2620
NanoBRET™ TE Intracellular Kinase Assay K-9	100 assays	N2630
NanoBRET™ TE Intracellular Kinase Assay K-10	100 assays	N2640

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NanoBRET™ TE Intracellular Kinase Assays (continued)

Product	Size	Cat.#
NanoBRET™ TE Intracellular Kinase Assay K-11	100 assays	N2650

Additional assay sizes are available. For a complete listing of the available kinase TE expression vectors and their NanoBRET™ tracer compatibility, visit: **www.promega.com/kinasevectors**

NanoBRET™ TE Nano-Glo® Substrate/Inhibitors

Product	Size	Cat.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	100 assays	N2162
Intracellular TE Nano-Glo® Substrate/Inhibitor	1,000 assays	N2160
Intracellular TE Nano-Glo® Substrate/Inhibitor	10,000 assays	N2161
Intracellular TE Nano-Glo® Vivazine™ Inhibitor	1,000 assays	N2200
Intracellular TE Nano-Glo® Vivazine™ Inhibitor	10,000 assays	N2201

Additional NanoBRET™ Target Engagement Assays

Product	Size	Cat.#
NanoBRET™ Target Engagement Intracellular HDAC Assays	100 assays	N2080
NanoBRET™ TE Intracellular HDAC Complete Kit	1,000 assays	N2170
NanoBRET™ Target Engagement Intracellular BET BRD Assays	100 assays	N2131
NanoBRET™ TE Intracellular BET BRD Complete Kit	1,000 assays	N2180
NanoBRET™ TE Intracellular E3 Ligase Assay, CRBN	100 assays	N2910
NanoBRET™ TE Intracellular E3 Ligase Assay, VHL	100 assays	N2930

Additional assay sizes are available.

Transfection Reagents and Accessories

Product	Size	Cat.#
FuGENE® HD Transfection Reagent	1ml	E2311
Transfection Carrier DNA	5 × 20µg	E4881

Additional sizes are available.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

For Research Use Only. Not for Use in Diagnostic Procedures

[a] **NanoBRET™ TE Selectivity Vector Panel Limited Use Label License**

BY USE OF THIS PRODUCT, USER AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE LABEL LICENSE. If user is not willing to accept the terms of this label license, and the product is unused, Promega will accept return of the unused product and provide user with a full refund.

User may use this product for research use only; however, no amplification or isolation of the vector components of this product and no transfer or commercial use of this product is allowed. Commercial use means any and all uses of this product by a party in exchange for consideration, including, but not limited to: (1) use in further product manufacture; and (2) resale of the product, whether or not such product is resold for use in research. User shall have no right to manufacture or have a third party manufacture the vector components within this product for any purpose. User shall have no right to modify or otherwise create variations of the nucleotide sequence of the luciferase gene. Notwithstanding the foregoing, user may use this product in provision of services to third parties in exchange for consideration; however, user may not transfer the product to third parties. No other use of this product is authorized without the prior express written consent of Promega.

In addition, user must:

(1a) use Nano-Glo®-branded luminescent assay reagents (LARs) for all determinations of luminescence activity of this product; or

(1b) contact Promega to obtain a license for use of the product with LARs not manufactured by Promega.

For uses of this product for energy transfer (such as bioluminescence resonance energy transfer), user must:

(2a) use NanoBRET™-branded energy acceptors (e.g., NanoBRET™ tracers, NanoBRET™ dyes, BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity; and

(2b) use NanoBRET™-branded LARs (e.g., NanoBRET™ Nano-Glo® Substrate) or Intracellular TE Nano-Glo® Substrate/Inhibitor or Intracellular TE Nano-Glo® Vivazine™/Inhibitor for all determinations of luminescent activity; or

(2c) contact Promega to obtain a license for use of the product for energy transfer assays using energy acceptors not manufactured by Promega. No license is needed if the energy transfer acceptor is a genetically encoded autofluorescent protein.

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